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EARLY EFFECTS ON THE MORPHOLOGY OF MOUSE SMALL INTESTINE OF SINGLE OR COMBINED MODALITY TREATMENT WITH HYPERTHERMIA AND X-IRRADIATION

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Abstract

This study describes the effects of hyperthermia and X-irradiation on the morphological appearance of normal, at risk tissues in the ileum of the mouse. The early morphological effects 1 day after a combined modality treatment are compared with those due to either hyperthermia or X-irradiation given alone. The response was assessed qualitatively and semiquantitatively using scanning electron microscopy and a villous scoring technique.

Early post-irradiation effects on topography did not differ significantly from those observed after small intestine exteriorisation without treatment. The villous scores for the combined modality treatments reflected greater damage than would be expected from the sum of villous scores for each modality treatment on its own. This suggests that the combined modality treatment had a synergistic or enhancing effect. A 4 hour time interval between the two treatments did not seem to reduce the enhancing effect. Further studies are required to investigate the effects of fractionated combined treatment.

KEY WORDS: Hyperthermia, intestine, mouse ileum, partial body X-irradiation, scanning electron microscopy, villous classification

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Introduction

Hyperthermia can be combined with different forms of radiation (Hornback et al., 1977; Kaplan, 1977; Kim et al., 1977, 1978) as in the management of neoplastic disease. Noteworthy recent review articles on this subject include those on the use of hyperthermia in cancer treatment by Hynynen and Lulu (1990) and on tumour control in long-term survivors following superficial hyperthermia by Myerson et al (1990). The reported improvement in therapeutic response and remission periods promises increased usage of this form of treatment for different types of cancer. The effects, and their underlying mechanisms, on at risk normal structures need, however, to be investigated. The present study investigates these possible effects on normal, at risk tissues as exemplified by the mouse small intestine. It aims mainly at qualitative and quantitative assessment of the extent of the structural alterations produced by combined treatment compared with those induced by each modality singly.

The structure of the normal small intestinal mucosa is vulnerable during treatment of pelvic or abdominal tumours. Since this tissue forms a unique blend of cell types with different anatomical locations and kinetic turnover rates, it provides an ideal model for investigation of *in situ* morphological effects of various agents on different phases of the cell cycle (Potten, 1981, 1982; Potten et al., 1983). Previous studies have described the effects of hyperthermia and/or irradiation on the small intestinal mucosa (Withers and Elkind, 1970; Carr and Toner, 1972; Anderson and Withers, 1973; Merino et al., 1978; Hume et al., 1979; Carr et al., 1982, 1983, 1984; Milligan et al., 1984; Tsubouchi et al., 1984; Hume and Marigold, 1986; Wyatt et al., 1987 and Kamel et al., 1988) and a variation in villous shape has been reported following different treatment procedures. Carr (1981) found progressive morphological alterations post-irradiation which suggested that the finger-shaped control villi passed through various stages of villous collapse. These shape changes provide the

basis of a score system for villous classification (Carr et al., 1983) which is applied in the present investigation to assess observed topographical alterations in a semiquantitative manner.

Materials and Methods

Animal model

Twelve week old female HC:CFLP mice (Hacking and Churchill, Ltd) were used in all experiments. Animals were maintained in an air-conditioned room operating on a 12 hour light and dark cycle and fed on a diet of Labsure TRD. Each group contained four animals and all groups were age related. All mice except group 1 'control' animals were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (Sagatal) at 0.06 mg/g body weight. The experimental groups were as shown below.

1) Control Animals received no treatment.
2) Anaesthetised control Animals were placed in the irradiation jig and then allowed to recover. i.e. sham-irradiated.
3) Exteriorised control The small intestine from the duodenum to the caecum was exteriorised and immersed in Krebs-Ringer solution at 37°C (pH 7.4, with Benzylpenicillin 1200 mg/l and Streptomycin sulphate BP 2 g/l) for 1 hour. The Krebs-Ringer solution was contained in a small perspex bath which was itself heated by partial submersion in a thermostatically controlled water bath. The equilibrium time of the intestine with the bath temperature was very rapid and of the order of 20 seconds. After treatment the intestine was replaced and the wound sutured. Full details of the heating procedure and temperature monitoring have been given elsewhere (Hume et al., 1979).
4) Irradiation The abdomen was exposed ventro-dorsally to 9 Gy X rays. The remainder of the animal was shielded with 3 mm-thick lead. Dose rate = 1.5 Gy/min. Pantak X-ray machine:- 240 kVp; filtration 0.25 mm Cu, 1.00 mm Al; HVL 1.12 mm Cu.
5) Hyperthermia Animals were treated as for the exteriorised control group 3 excepting that the bath temperature was 42.0°C.

Combined Modality

6) (H-I) Irradiation given within 5 minutes of completion of hyperthermia.
7) (E-I) Irradiation given within 5 minutes of completion of exteriorisation.
8) (H-4hr-I) Irradiation given 4 hours after hyperthermia completed.

After the exteriorization treatment, in the case of all the combined modality treatments, the intestine was replaced and the wound sutured before subsequent irradiation. The various treatments were assessed qualitatively and semiquantitatively after the application of scanning electron microscopy (SEM).

Specimen sampling

Twenty four hours after each treatment, the animals were killed by cervical dislocation. Samples of ileum were preserved by immersion fixation in 5% glutaraldehyde in Millonig's phosphate buffer solution at 4°C (Carr and Toner, 1972). The fixative was used to inflate the gut to physiological size and then the ends of each sample were tied with thread.

Specimen preparation for scanning electron microscopy (SEM)

After glutaraldehyde fixation, the inflated gut samples were opened longitudinally, pinned onto a piece of cork and rinsed in 0.2M sodium cacodylate buffer for 2 hours. The specimens were washed in distilled water for 5 min, dehydrated in an ascending series of ethanol and critical point dried using liquid CO_2, as the transitional fluid, in a Polaron E3100 Jumbo critical point dryer. Tissues were then mounted on aluminium stubs and coated with gold/palladium for 2 min using a Polaron P300 sputter-coater.

The samples were examined in a JEOL 840 scanning electron microscope. The entire area of each specimen (measuring approximately 1.5 cm x 1 cm) was examined and images recorded at various magnifications. Images for SEM montages of the whole specimen area were prepared at a fixed magnification (x 35).

TABLE 1 Villous score classification

Villous classification	Score
Normal, erect (VE) or laterally collapsed villi (VL)	0
Vertically collapsed villi (VV)	1
Horizontal villi (VH)	1
Conical villi (VC)	3.5
Rudimentary villi (VR)	6
Absent villi (VA)	8.5
Ulceration	10

Semiquantitative analysis of montages (Villous Scoring)

The stages of villous collapse (Figure 1, Table 1) were classified as follows: (a) lateral villous collapse (VL) - villous leans or bends away from its normal erect posture, (b) vertical villous collapse (VV) - villi remain erect, but show prominent creasing patterns and separation of villi, (c) horizontal villous collapse (VH) - the villi have collapsed entirely to one side, (d) conical villi (VC) - the villi are conical in shape, with broad bases and narrow tips, (e) rudimentary villi (VR) - villi have lost almost all of their original shape and (f) flattened mucosa and absence of villi, (VA) - only very few mounds, barely reminiscent of villi, may be seen.

Villous scoring assessed by a grid overlay technique

This procedure (Carr et al., 1983; Indran et al., 1985), utilised an 18 cm x 26 cm transparent acetate 'overlay' sheet which was sub-divided into a grid composed of 1 cm

squares. The grid overlay approximated the same size as the individual specimen areas depicted on each montage of micrographs. An assessment was made of villous shape for the one to three villi which were present in each grid square. An overall count was made of the total number of squares which shared the same villous classificiation. Each count was then multiplied by the score relevant to that villous shape classification to give a subtotal. All subtotals were added and then divided by the total number of squares to obtain the final specimen score. The scores calculated for each animal were used to obtain an overall score for each experimental group. It should be noted that the higher the score value then the greater the extent of tissue damage and deviation from the norm.

The scoring procedure was carried out blind and assessed three times for each specimen and, in addition, spot checks were made on the scores by another observer. The grid score data was statistically examined for intergroup significance (0.05 significance level) using the Student-Newman-Keuls statistical test.

Results

Qualitative analysis

An appreciation of some qualitative responses of the ileum of the small intestine of HC:CFLP mice to various treatments may be gained from Figure 2. The normal erect appearance of the intestinal villi found in the untreated control group contrasts with that of experimental groups receiving X-irradiation either singly or in combination, where villi of lower profiles less suited to absorption, such as conical and rudimentary villi were present, the latter only in group 6. The most obvious qualitative topographical alterations were manifested in the ileum of group 8 when conical villi were especially evident. Horizontal villi (VH), absent villi (VA) and ulcerations were the only structural abberations not detected. Transverse and longitudinal villous creasing was more prominent, especially in combined modality treatments, while localized bulging was also apparent in the case of hyperthermia treatment (group 5).

Variable numbers of rod-shaped bacteria were present on villi throughout all treatment groups. Goblet cells were especially prominent in all combined modality treatments. The intervillous basin was not readily observed in control groups but was easily seen in non-control groups. Crypt openings were narrow and unremarkable in all instances except in group 7 animals in which they were occasionally wide. Microvilli displayed a regular compact pattern although groups 5 and 6 had button shaped microvilli interspersed with this pattern.

Semiquantitative analysis of data

No statistically significant differences in score values were found when the various groups listed separately within either A, B or C below were compared (Table 2). A - control (group 1) and anaesthetized control (group 2); B - exteriorised control (group 3), X-irradiation (group 4) and hyperthermia (group 5); C - hyperthermia then X-irradiation 5 min later (group 6), exteriorization then X-irradiation 5 min later (group 7, and hyperthermia then X-irradiation 4 hours later (group 8). However statistically significant differences in score values were found when the groupings A, B and C were compared. Basically, the C groups (or combined modality experiments) revealed the greatest extent of villous damage (reflected in their higher score values), the B groups showed an intermediate level of damage and the A groups, which included the control groups 1 and 2 acted as a base line against which other groups could be compared.

TABLE 2 SEM score values for different experimental groups

Experimental groups	Score value	± standard deviation	
(1) Control	0.09	± 0.44	A*
(2) Anaesthetised control	0.1	± 0.33	A
(3) Exteriorised control	0.5	± 0.26	B
(4) X-irradiation	0.7	± 0.35	B
(5) Hyperthermia	1.0	± 0.02	B
Combined modality			
(6) Hyperthermia, then X-irradiation 5 min later	1.8	± 0.48	C
(7) Exteriorisation then X-irradiation 5 min later	2.28	± 0.06	C
(8) Hyperthermia, then X-irradiation 4h later	2.81	± 0.08	C

* Grouping A, B and C based on villous collapse

Discussion

The use of hyperthermia and X-irradiation as a combined therapy for treatment of cancer has been based on early reports of hyperthermal induced enhancement of the cellular response to ionizing radiation (Belli and Bonte, 1963). Experimental studies showed that tumours in accessible sites in dogs and cats were treated successfully with a combination of hyperthermia and radiotherapy (Miller et al., 1977). The potential application of such combined modality treatment has been explored in a variety of

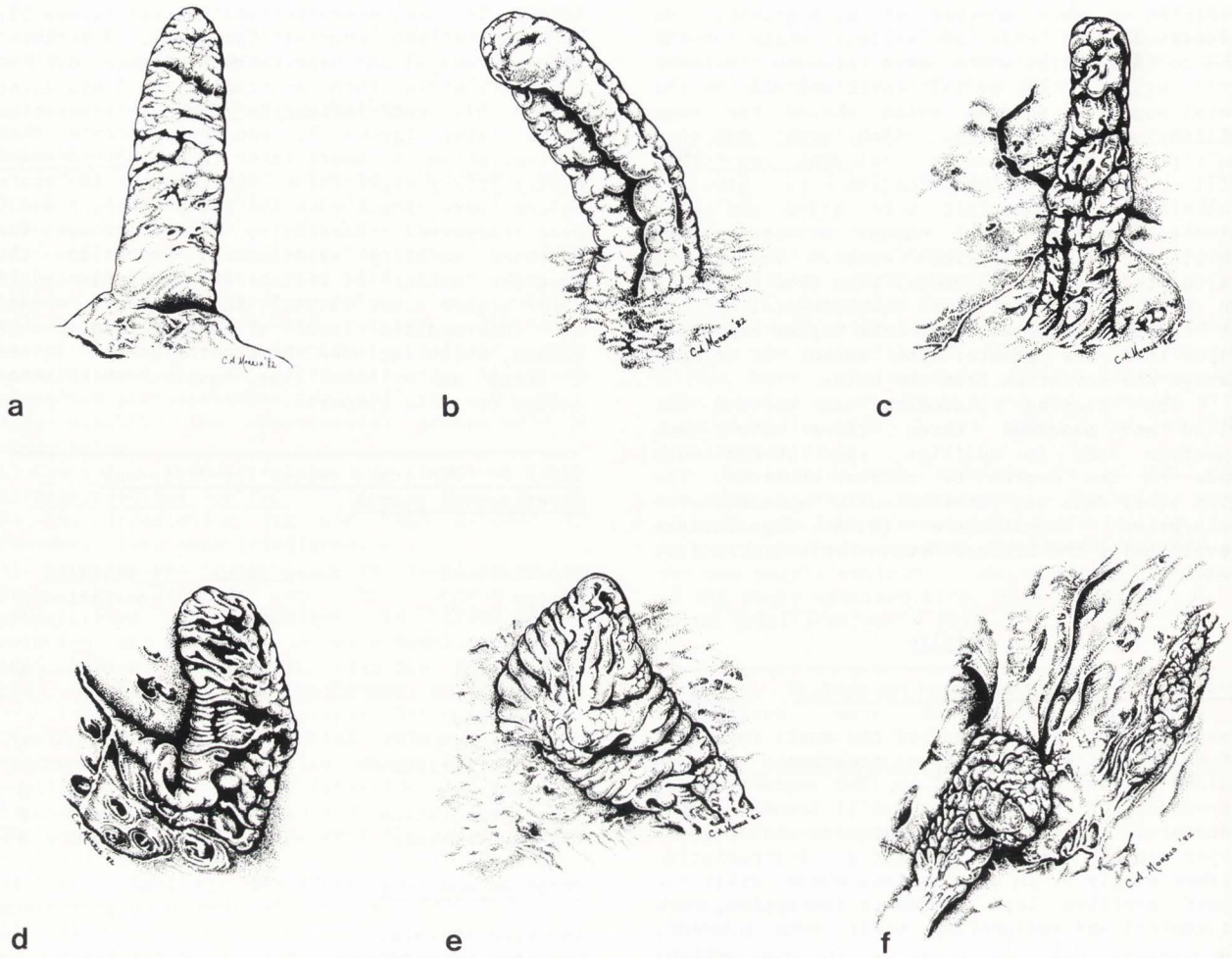

Figure 1. Diagrammatic representations of a) a normal erect control villus and (b-g) various stages of villous collapse; b) lateral collapse c) vertical collapse d) horizontal collapse e) conical collapse f) rudimentary collapse and g) villi absent. Note: all types of villous collapse were not detected in this investigation.

malignant neoplasms (Hornback et al., 1977; Kaplan, 1977; and Kim et al., 1977, 1978). Since in radiotherapy the effects on normal tissues is dose-limiting, the interaction of hyperthermic and radiation damage in these tissues must be understood so that each modality can be used correctly in tumour therapy (Milligan et al., 1984). Fajardo's (1984) review of the pathological effects of whole-body and localized hyperthermia on a variety of tissues clearly indicated the need for more information in order to define the range of safety for clinical hyperthermia.

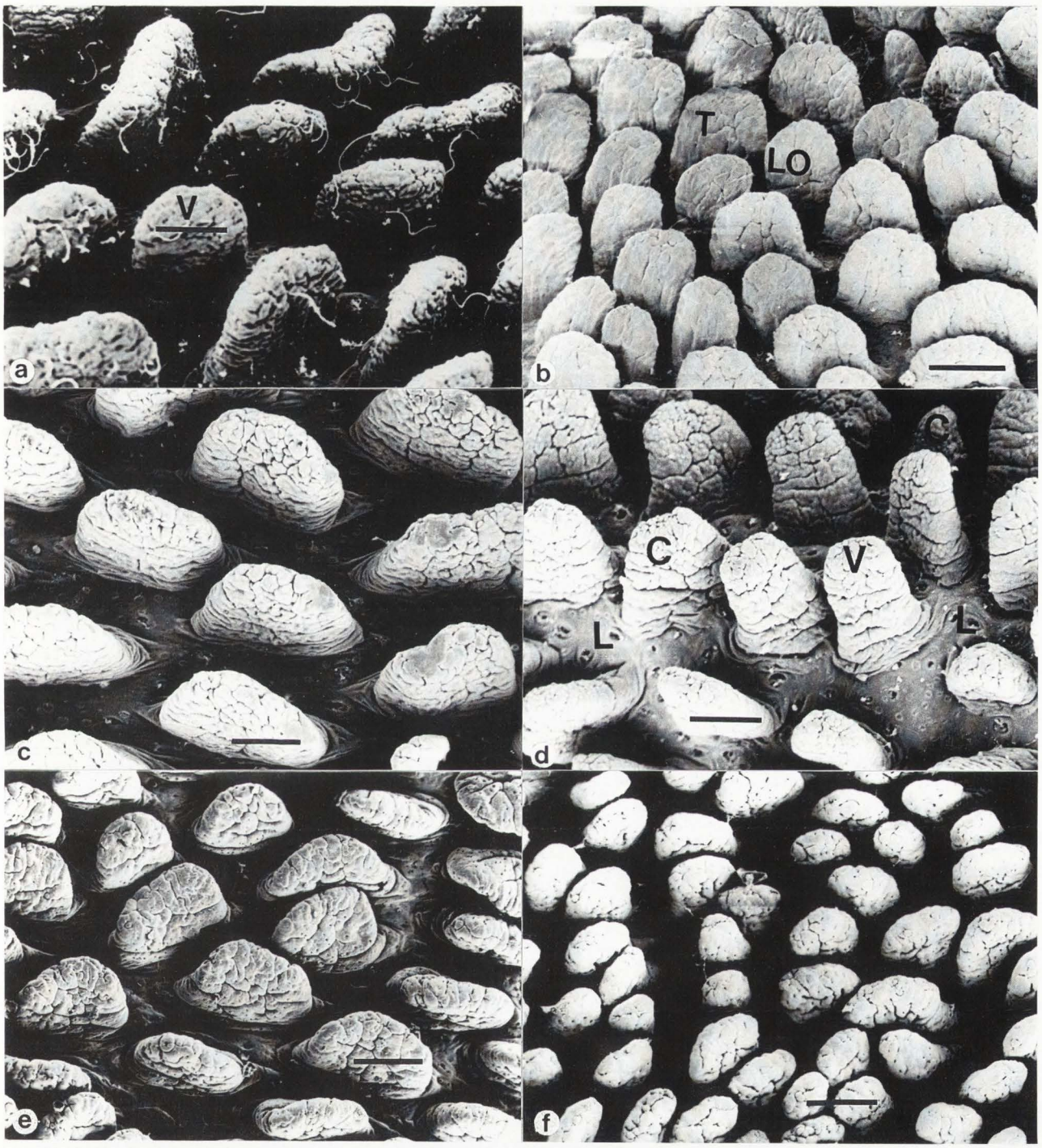

Figure 2. Scanning electron micrographs of mouse small intestinal villi, 24 hours after the treatments described below. (2a) Group 1 (untreated control) showing normal erect villi (V); 2b) Group 3 (exteriorised control). The villi, which are erect, show transverse (T) and longitudinal (Lo) creases; 2c) Group 4 (X-irradiation). Creases are prominent. Crypt mouths are clearly seen between villi of different appearances; 2d) Group 7 (X-irradiation within 5 min of completion of exteriorization at 37°C). Vertical (V) and conical (C) villi are observed. Prominent creases are seen on the villous surface. Crypts (L) with widened lumina are present between the villi; 2e) Group 6 (hyperthermia at 42°C followed 5 min later by X-irradiation), conical villi are shown. 2f) Group 8 (hyperthermia at 42°C followed 4 hours later by X-irradiation). Villi of different shapes with prominent creases are present. Scale bars = 100 μm.

Only a few studies have described the effects of combined hyperthermia and irradiation on the normal small intestine (Milligan et al., 1984; Hume and Marigold, 1985, 1986). The methods used for assessing the tissue response to combined treatment have included animal survival studies, crypt survival counting and crypt stem cell survival data. No previous study, however, has described the effects of combined hyperthermia and irradiation on the mucosal surface of the mouse small intestine. Kamel et al. (1988) assessed the damage to the mouse small intestine in the period up to 24 hours after heating a portion of the gut for 20 min at 43°C and also in the period up to 9 days after 10 Gy whole body X-irradiation using SEM and a semi-quantitative scoring system. Their results indicate that at 24 hour time points there were minimal or slight variations in comparison with those due to hyperthermia at earlier time points (in particular 2 and 4 hours) or to those due to irradiation at later time points (in particular 3 and 5 days).

The data described in this paper compares the effects of single and combined modality treatments and can be subdivided into qualitative and quantitative changes. Each will be discussed in turn.

Qualitative Changes

The various experimental groups, 24 hours after insult to the mouse ileum, revealed a variable shape deviation (reflected in SEM score values, Table 2) from the untreated control pattern of erect villi. The prominence of goblet cells in the ileum after combined modality treatments is similar to findings reported in the mouse jejunum at the 1 day time period after exposure to 10 Gy of X-irradiation (Kamel 1986) but the underlying mechanism for this effect is not known.

Quantitative Changes

Semiquantitative investigation of the topographical appearance of the mouse ileum 24 hours after exposure to insult revealed that the expression of villous score damage enables distinction of combined modality treatments (highest score values) from single modality treatments (intermediate score values) and in turn from baseline control groups (lowest score values). Villous shape changes assessed by the villous scoring technique have been registered at 1 day after treatment with 5 Gy whole body neutron irradiation as 0.8 $\pm$ S.D. 0.26 (Carr et al., 1992). This is comparable to the score value of 0.7 $\pm$ S.D. 0.35 found in the present investigation at 1 day using 9 Gy X-irradiation. This villous score data is also similar to that recorded previously (Carr et al., 1984; Indran et al., 1985, 1991). It is encouraging that the use of novel morphological index data (Carr et al., 1991, 1992) demonstrates a similar damage pattern to that described here using villous score assessment. This recent work also claims that not only is villous shape change accompanied by changes in epithelial cell shape and stromal changes but is most closely linked to alterations in the smooth muscle component of the gut (Carr et al., 1992).

As far as the observation of topographical features is concerned the present result may suggest that single modality early post-irradiation and hyperthermia effects are not very much different from those after surgery. It is significant that combined modality group 7 (exteriorised, then X-irradiated) had a much higher score value than group 4 (X-irradiated without prior exteriorisation). This finding highlights the traumatic effect of the operative manipulation which could enhance the severity of post-operative radiotherapy. It also suggests that there was no significant contribution by hyperthermia (42°C) to the score value at the 24 hr time point which agrees with the findings of Kamel et al. (1988) who showed recovery of villi from hyperthermia (43°C) at this time point. Since the 4 hour time interval between doses of hyperthermia and X-irradiation in group 8 did not affect the enhancing effect of the combined treatment, this indicates that neither partial recovery from the initial insult nor increased tolerance to the subsequent insult, even if they did occur, had contributed any protective role from the subsequent but different form of insult.

In summary, this study suggests that combined modality treatment has an enhancing effect on some structural aspects of the ileum of the mouse small intestinal mucosa at the 24 hr time point after exposure to insult. It would therefore be interesting to investigate the score values of combined modality treatment when hyperthermic insult follows the irradiation. Thermal enhancement of the tissue response to irradiation could then be assayed at the time of expression of radiation injury.

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Discussion with Reviewers

L.G. Friberg: You have chosen to look at the effect after 9 Gy, single dose, when 24 hours has gone. This timing could maybe be questioned. It should be of interest to look after irradiation effect when it is optimal. Usually macroscopical effect does not be measurable within 48 hours after irradiation and after 9 Gy optimal effect seen by SEM comes after 72 hours. Why did you choose 24 hours?

M. Albertsson: The results were observed on only one occasion, 24 hours after treatment; why just then in particular? And why not more than one occasion - multiple observations would have added weight to the findings. Query: What

was the guiding principle in the choice of intervals for combination treatment?
Authors: We agree that previous publications indicate that the structural effects of 9 Gy are optimally seen after 72 hours and that the effects of hyperthermia are less prominent and even negligible by 24 hours. It was of interest to investigate whether the expected synergistic effect of combined treatment would present a structural effect at 24 hours either through delaying recovery from the heat treatment through a radiation effect or accelerating the effects of radiation by the hyperthermia treatment. Since thermal enhancement of radiation is reduced as the interval between treatments is increased then we could have assayed at the known time of expression of radiation injury (72 hours), with a 4 hour temporal separation of modalities and quantified the thermal enhancement of radiation injury at that point.

L.G. Friberg: The result of a single dose irradiation on the small intestine is both a direct one with disturbance of physiology and also an indirect one with decrease of new cell production in the crypts followed later by cone-formed and shorter villi. This can be measured with a score system. Hyperthermy gives a damage but also increased blood flow. Have you studied the effect of increased blood flow? In this study there were no difference between hyperthermy and exteriorisation or irradiation itself after 24 hours. Maybe there were effects that could be seen on higher magnification?
Authors: With regard to the first point, we did not study the effect of increased blood flow. With regard to the second point, we agree that some effects may or may not be seen on higher magnification. However, the applied score system cannot be suitably used at higher magnification. It would certainly be of interest to compare any changes that may occur at higher magnification.

L.G. Friberg: Is it reasonable that combined treatment will give a greater damage? If radiobiological studies shall contribute in radiotherapy you have to simulate a technique usable in the clinic. How do you give hyperthermy to the patients small intestine?
Authors: The aim was not to simulate a technique usable in the clinic. It was to study the morphological effects of combined modality therapy on normal body structure. However, it should be noted that the hyperthermic temperature of 42°C was chosen because of its clinical relevance in whole or partial body hyperthermia, patients cannot tolerate a core temperature above this.

Reviewer III: The authors use a (semi-)quantitative approach to classify changes in villous structure. The "score" assigned to each type of change (Table 1) can hardly be exact. Why are conical villi 3.5 and not 3, or 2.5, or 5? With a different choice for the score, differences that now are significant might become not significant, and the other way round. Couldn't the data be presented in a table classifying the frequency of each group. Also with this way of presenting the data it would be possible to check for statistical significance of differences between groups.
Authors: Progressive alterations in villous shape (Carr et al., 1983; Indran et al., 1985) have been scored on a scale from 0 to 10. The villous collapse imaged by the scanning electron microscope has been linked with the results of histometric studies of rat mucosa by Altman (1974). His technique involved drawing outlines of villous areas from projections of microscopic images. Planimetry was used to measure villous area and the results were plotted as a graph of area against time after a 90 Gy dose of abdominal X-irradiation. That similar progressive villous collapse occurred in mice is confirmed by early work with lethal doses of 15-25 Gy gamma radiation (Carr and Toner, 1972) and it is notable that at higher doses each stage of damage is seen sooner. Altman calculated the drop in average villous area (post-irradiation) on a day to day basis and the resultant ratio approximated 1:2.5:2.5. This ratio was used (Carr et al., 1983) in the construction of a scale, applied in the current work for assessing villous damage by scanning electron microscopy. Erect or control (VE and VL) villi are scored as 0, since VL structures occur in control animals and since it is presumed that the area is the same. VV villi are scored as 1, VH villi are also scored as 1, VC villi are scored as 1 + 2.5 = 3.5, VR villi are scored as 3.5 + 2.5 = 6.0, VA has been scored as 6.0 + 2.5 = 8.5 by extrapolation and a cut-off score of 10 is used for ulceration. The progressive damage ratio therefore determines why conical villi are scored at 3.5 and not at another value.

The final specimen score (see Materials and Methods section) is influenced by an aggregation of sub-totals derived from the various villous types found across the entire specimen area under investigation and reflects the overall degree of villous damage observed. By application of this technique we found statistically significant differences between groups but appreciate that alternative methods of presenting the data also make it possible to check for statistically significant differences between groups.

Reviewer III: How does the nomenclature in the figure legends (vertical, longitudinal creases) compare to the classification of Table 1? How do the crypts with widened lumina affect the score?
Authors: The occurrence of vertical and longitudinal creases on villi is not included in the score system and is mentioned as a qualitative observation. The score system does not take account of changes that occur in the crypts.